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The interaction between E-tropomodulin and thymosin β-10 rescues tumor cells from thymosin β-10 mediated apoptosis by restoring actin architecture

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Abstract **Thymosin β-10 (TB10) is a small G-actin binding protein that induces depolymerization of intracellular F-actin pools by sequestering actin monomers. Previously, we demonstrated that overexpression of TB10 in ovarian tumor cells increased the rate of cell death. As an initial step to define molecular mechanism of TB10-dependent apoptotic process in ovarian tumor cells, we searched a human ovary cDNA library for a novel TB10 binding protein using a yeast two-hybrid system. The selected protein was human E-tropomodulin (E-Tmod), another component of the actin binding proteins. Subsequently, two interacting protein components were determined quantitatively. Results showed that the full-length TB10 is required to bind with E-Tmod, and the TB10 binding site on E-Tmod partially overlaps with the actin binding site on E-Tmod. Moreover, introduction of E-Tmod cDNA into a tumor cell line reversed TB10 mediated apoptosis and restored actin architectures. These results may suggest that TB10 regulates apoptotic homeostasis by not only just binding to actin but also competing or blocking the protein complex formation of E-Tmod with actin.**

Key words: Actin sequestering protein; Apoptosis; Thymosin β-10; E-tropomodulin; Yeast two-hybrid

1. Introduction

Actin is one of the primary proteins that are responsible for maintaining the structure of most eukaryotic cytoskeletons [1]. The dynamic equilibrium between monomeric G-actin and F-actin regulates many cellular functions ranging from mitosis to motility. It is clear that such functions depend on the tight control of actin polymerization by actin monomer sequestering proteins. The β-thymosins are a family of highly conserved polar 5 kDa peptides. Both in vitro and in vivo studies indicate that they bind monomeric G-actin and prevent actin polymerization into F-actin in the cytoplasm of non-muscle cells [2].

Thymosin β-10 (TB10) is a 5 kDa polypeptide comprised of 43 amino acid residues containing an unusual acetylated alanine as the first amino acid residue in a mature peptide [2]. TB10 and thymosin β-4 (TB4), another member of the thymosin family, are major G-actin sequestering proteins in mammalian tissues. Depending on the concentration of TB10 or TB4, intracellular F-actin networks are selectively increased or decreased [3–5].

Two critical outcomes for mammalian cells may be affected by the action of TB10: programmed cell death [6] and metastasis [7]. In human carcinogenesis, the β-thymosins bind and sequester G-actin, which plays a pivotal role in maintaining the cellular cytoskeleton. The perturbed balance between monomeric G- and F-actin in transformed cells alters the motility and adhesion processes that are important in tumor progression and metastasis formation as well as apoptosis.

Whereas TB10 may contribute to invasion or metastasis in some cancer types, we demonstrated its involvement in apoptosis of ovarian tissue [8]. Overexpression of TB10 in ovarian tumor cells increased the rate of cell death [8]. Similar to this observation, thymosin β-9 overexpression also altered the level of apoptosis in infected bovine macrophages [9].

To begin characterization of the TB10-dependent apoptotic pathway, we used a yeast two-hybrid system to screen a human ovary cDNA library for novel TB10 binding proteins. We identified human E-tropomodulin (E-Tmod), another component of the actin binding proteins. Interestingly, TB10 and actin share the same binding site on E-Tmod. Transient transfection analyses demonstrated that increased E-Tmod expression efficiently blocks the TB10-dependent apoptotic activity. Therefore, these results support that a dynamic equilibrium among pools of actin, TB10 and E-Tmod regulates an apoptotic homeostasis.

2. Materials and methods

2.1. Yeast two-hybrid screening

For bait construction with human TB10, cDNA encoding full-length human TB10 (Met^{-1}-Ser^{43}) was subcloned into the *Eco*RI and *Sal*I restriction sites of the pGilda. The resulting plasmid pGilda-TB10 was introduced into yeast strain EGY48 [*MATa*, *his3*, *trp1*, *ura3-52*, *leu2::pLeu2-LexAop6*/pSH18-34 (*LexAop-lacZ* reporter)] by

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Abbreviations: ICE, interleukin-I converting; TB10, thymosin β-10; E-Tmod, E-tropomodulin; TB4, thymosin β-4

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a modified lithium acetate method [10]. The ovary cDNA library was constructed by cloning the cDNA fragments into the *Eco*RI and *Xho*I restriction sites of the pJG4-5 to generate B42 fusion proteins (Clontech, Palo Alto, CA, USA). The cDNAs encoding B42 fusion proteins were introduced into the competent yeast cells that already contained pGilda-TB10 and the transformants were selected for the tryptophan prototrophy (plasmid marker) on synthetic medium (Ura^-, His^-, Trp^-) containing 2% glucose.

2.2. Quantitation of interaction

The activity of the interaction between TB10 and E-Tmod was compared by measuring the relative expression level of β-galactosidase. The β-galactosidase assay was determined according to the previously described method [11] with slight modification. Briefly, yeast cells (EGY48 strain) containing each construct were cultured in yeast synthetic media (Ura^-, His^-, Trp^-) with 2% glucose until they reached the mid-log phase. Then, the cells were transferred in a yeast medium (Ura^-, His^-, Trp^-) containing 2% galactose and 0.2% Me_2SO. Equivalent numbers of cells were lysed in 0.7 ml Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol, pH 7.0) containing 50 μl 0.1% sodium dodecyl sulfate (SDS) and 50 μl chloroform for 30 s at 30°C. Then, β-galactosidase activity was measured by adding 140 μl of 4 mg/ml *o*-nitrophenyl β-D-galactopyranoside (ONPG). The reaction was carried out at 30°C until yellow color appeared, and then quenched by adding 0.4 ml of 1 M Na_2CO_3. The samples were centrifuged briefly to remove cell debris, and the absorbance was measured at 420 and 550 nm. The β-galactosidase activity was calculated using the formula units = $[1000 \times (A_{420} - 1.75 \times A_{550})]/(\text{time} \times \text{volume} \times A_{600})$.

2.3. In vitro pull-down assay

cDNA encoding human TB10 was subcloned into the *Eco*RI and *Xho*I restriction sites of the pGEX4T-1 (Amersham Biosciences, Uppsala, Sweden) to generate glutathione *S*-transferase (GST) fusion protein (GST-TB10). The human E-Tmod and actin cDNA were ligated into pET29a (Novagen, Madison, WI, USA) using *Eco*RI and *Xho*I to generate a histidine fusion protein (His-E-Tmod and His-actin). GST and histidine fusion proteins were purified using either GST column (Amersham) or Ni^{2+} column (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocols. The purified GST-TB10 (2 μg) was mixed either with His-E-Tmod (2 μg) or with His-actin (2 μg), or both of them. The mixtures were then added to 20 μl of GST column matrix (glutathione Sepharose 4B) and incubated at 25°C for 30 min. The slurry was pelleted by centrifugation and washed. The pellet of the gel matrix was resuspended in 20 μl elution buffer (10 mM glutathione, 50 mM Tris–HCl, pH 8.0) and incubated at 25°C for 20 min to elute the bound GST fusion proteins. The eluted proteins were separated by gel electrophoresis and the proteins were detected by Coomassie staining.

2.4. Subcloning of deletion mutants of TB10 and E-Tmod

Two deletion mutants (Δ-1–28 and Δ29–43) of TB10 were isolated by polymerase chain reaction (PCR) using the combination of the following primers (TB-F1, 5′-ATTGAATTCATGGCAGACAAACCAGAC-3′; TB-R1, 5′-ATTCTCGAGTTACAGGGTGTTCTTCTC-3′; TB-F2, 5′-CCGGAATTCCCGACCAAAGAGACCATT-3′; TB-R2, 5′-CCGCTCGAGTTAGGAAATTTCACTCCG-3′). PCR products spanning each fragment were cloned into the *Eco*RI and *Xho*I restriction sites of the pGilda.

Three deletion mutants (Δ1–161, Δ1–230 and Δ1–310) of E-Tmod were isolated by PCR using the combination of the following primers (E-Tmod-F1, 5′-CCGGAATCACGATGTCGTACAGACGAGAAC-3′; E-Tmod-F2, 5′-CCGGAATTCAACAGCGTGATTAAACCCACA-3′; E-Tmod-F3, 5′-CCGGAATTCATCGTGGGGACACGGAGTAAT-3′; E-Tmod-F4, 5′-CCGGAATTCACACTTCTCAAATTCGGCTAC-3′; E-Tmod-R1, 5′-CCGCTCGAGCTAGACACCACTCCGGCACTT-3′). PCR products spanning each fragment were cloned into the *Eco*RI and *Xho*I restriction sites of the pJG4-5. Each constructed plasmid was introduced into yeast EGY48 expressing either E-Tmod or TB10 hybrid protein.

2.5. Co-immunoprecipitation

cDNA encoding human TB10 (Met^{-1}-Ser^{43}) was isolated by PCR by using the specific template and then cloned into pEGFPC1 (Clontech) digested with *Bgl*II and *Eco*RI (pEGFPC1-TB10). The human E-Tmod cDNA (Met^{1}-Val^{359}) was ligated into pcDNA4/HisMax (Invitrogen) using *Eco*RI and *Xho*I (pcDNA4/HisMax-E-Tmod). For co-immunoprecipitation, SKOV-3 ovarian cancer cells were co-transfected with cDNA constructs of pEGFPC1-TB10 and pcDNA4/HisMax-E-Tmod using FuGENE6 (Roche Applied Science, Basel, Switzerland). As a negative control, pEGFPC1-TB10 and empty vector pcDNA4/HisMax was also co-transfected. Three days after transfection, cells were harvested by trypsinization and centrifugation. Cell pellets were washed in phosphate buffered saline (PBS), resuspended in cell lysis solution (50 mM Tris, pH 7.2, 150 mM NaCl, 1% Triton X-100, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 2 μg/ml aprotinin, 200 μg/ml phenylmethylsulfonyl fluoride). Lysates were incubated with anti-GFP antibody (Zymed) and precipitated with protein A-agarose. The precipitated proteins were resolved by SDS gel electrophoresis, transferred onto Immobilon P membrane (Millipore), and immunoblotted with anti-His antibody (Amersham) or rabbit anti-TB10 antiserum using the ECL system (Amersham).

2.6. Cell viability assay

SKOV-3 ovarian cancer cells were plated in six-well plates and co-transfected with different ratios of human TB10 and human E-Tmod cDNAs. Three days after transfection, cells were harvested, stained with trypan blue, and counted under the light microscope.

2.7. Apoptosis assay

SKOV-3 ovarian cancer cells were plated onto four-chamber slides and co-transfected with human TB10 and human E-Tmod cDNAs at ratio 1:1. Three days after transfection, chamber slides were rinsed with PBS, stained with 2 mg/ml of DAPI (Boehringer Mannheim, Mannheim, Germany) at 37°C for 15 min, washed twice with PBS and examined with the fluorescence microscope.

2.8. Fluorescence detection of F-actin

Cell monolayers were fixed for 40 min at room temperature with 4% paraformaldehyde in PBS and then stained with 25 μl/ml of phalloidin-FITC (Sigma, St. Louis, MO, USA) for 1 h in the dark. Stained cell monolayers were washed twice with 0.5% Triton X-100 in PBS. After washing, coverslips were mounted onto slides using a PBS/glycine mutant and examined with the fluorescence microscope.

3. Results

3.1. Identification of E-Tmod as a TB10 binding protein

The human ovary cDNA library fused to the gene for the transcription activator pJG4-5 was introduced into yeast cells containing the pGilda-TB10 (Met^{-1}-Ser^{43}) as bait. Approximately 3×10^6 independent transformants were pooled and respread on the selection media (Ura^-, His^-, Trp^-, Leu^-) containing 2% galactose to induce the expression of cDNA. If a B42 tagged protein interacts with the TB10, it will activate the transcription of *LEU2* gene and allow the host cells to grow on a synthetic medium lacking leucine. Among 15 colonies obtained on the selection media, a total of eight colonies showed galactose dependency. The plasmids were isolated from the selected yeast cells and introduced into *Escherichia coli* KC8 to isolate the plasmids carrying pJG4-5-cDNA inserts. The plasmids were then isolated by the plasmid marker *trp* in the *E. coli* host, and the purified plasmids were sequenced. A homology search in the GenBank using the BLAST program revealed that seven out of eight plasmids encoded E-Tmod. All of E-Tmod encoded plasmids contained a cDNA with 100% identity to the 291 amino acid C-terminal sequence of human E-Tmod (GenBank accession number: BC016731; Leu^{68}-Val^{359}; Fig. 1A). One positive plasmid was encoded human eukaryotic transcription factor 1-γ (GenBank accession number: BT006677).

The interaction between TB10 and E-Tmod was further examined by GST pull-down analysis and co-immunoprecipitation. For GST pull-down analysis, the GST-TB10 fusion

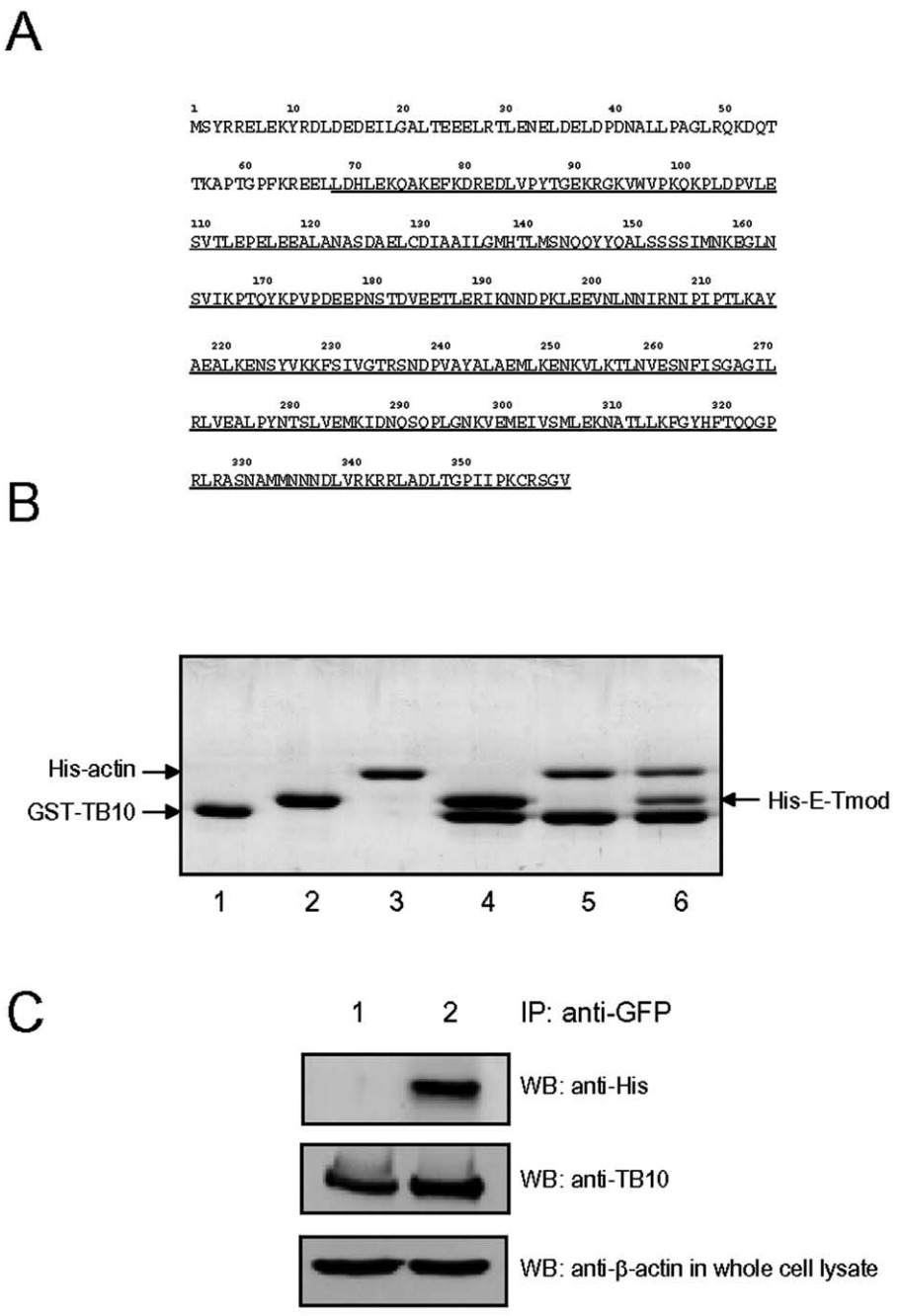

Fig. 1. Interaction analysis between human E-Tmod and TB10. A: The amino acid sequence of E-Tmod indicated using single letter abbreviations. The underlined amino acid sequence means the translated E-Tmod protein isolated from yeast two-hybrid screening. B: Interactions between TB10 (GST-TB10), E-Tmod (His-E-Tmod) and actin (His-actin) were confirmed by in vitro pull-down assays. For GST pull-down analysis, the purified GST-TB10 (2 μg) was mixed with either His-E-Tmod (2 μg) or His-actin (2 μg), or both of them. Then, the protein mixtures were subjected to GST affinity purification. The eluted proteins were separated by gel electrophoresis and the proteins were detected by Coomassie staining. Lanes: 1, GST-TB10; 2, His-E-Tmod; 3, His-actin; 4, GST-TB10 and His-E-Tmod; 5, GST-TB10 and His-actin; 6, GST-TB10, His-Tmod, and His-actin, respectively. C: Co-immunoprecipitation of TB-10 with E-Tmod. Lanes: 1, lysate from pEGFPC1-TB10 and pcDNA4/HisMax (vector only) co-transfectant; 2, lysate from pEGFPC1-TB10 and pcDNA4/HisMax-E-Tmod co-transfectant. IP means immunoprecipitation and WB means immunoblotting with indicated antibodies.

protein was mixed either with His-E-Tmod or with His-actin or both of them, the mixture was purified employing GST column matrix. The eluted proteins were separated by gel electrophoresis and the proteins were detected by Coomassie staining. As shown in Fig. 1B, GST-TB10 was co-purified with both His-E-Tmod and His-actin at approximately 1:1 molar ratio (lanes 4 and 5). When we mixed GST-TB10 with both His-E-Tmod and His-actin, we observed that all three proteins were associated, but amount of co-purified E-Tmod and actin by GST column matrix (lane 6) was relatively lower compared to that of E-Tmod with TB10 (lane 4) or that of actin with TB10 (lane 5). The molar ratio between co-purified E-Tmod and actin was at approximately 1:1 ratio in lane 6. This result may indicate that E-Tmod and actin com-

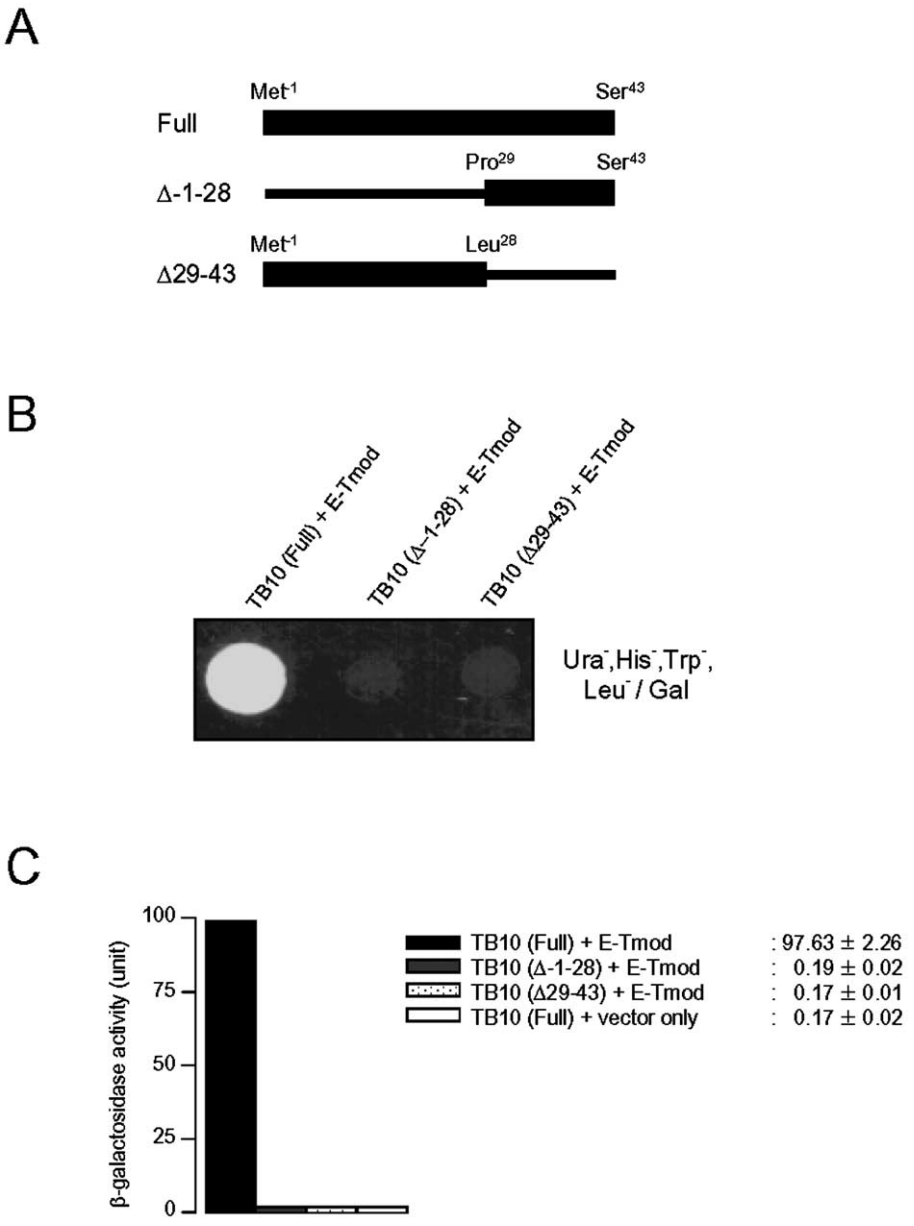

Fig. 2. Mapping the region of TB10 for interaction with E-Tmod. A: Schematic representation of cDNA constructs for each TB10 deletion mutant and full-length human TB10 fusion proteins in the yeast two-hybrid system. B: cDNA constructs were co-transformed into EGY48 yeast cells to test the protein–protein interaction within the yeast two-hybrid system. C: Binding activity of these constructs by ONPG assays. The results are representative of three separate experiments. Data are shown as mean ± S.E.M.

petitively bind to TB10. As positive controls, GST-TB10 (lane 1), His-E-Tmod (lane 2) and His-actin (lane 3) were purified using either GST column matrix or Ni^{2+} column and loaded, 200 ng each, on the gel. This result indicated that all of the three proteins form a complex, but the binding strength of each protein interaction remained to be determined.

For co-immunoprecipitation, cDNA constructs of TB10 (pEGFPC1-TB10) and E-Tmod (pcDNA4/HisMax-E-Tmod), or pEGFPC1-TB10 and pcDNA4/HisMax (vector only) were co-transfected into SKOV-3 ovarian cancer cells. Subsequently, an immunoprecipitation was performed using anti-GFP antibody with lysates from both transfected cells. After immunoprecipitation, the precipitated proteins were immunoblotted using anti-His antibody or anti-TB10 antibody. As shown in Fig. 1C, pcDNA4/HisMax-E-Tmod was co-immunoprecipitated with pEGFPC1-TB10 (lane 2 in the upper panel), whereas no interaction was observed between pcDNA4/HisMax (vector only) and pEGFPC1-TB10 (lane 1 in the upper panel). An immunoblotting using anti-TB10 antibody confirmed that an equal amount of TB10 was precipitated in both samples (middle panel). Whole cell lysates from both samples contained the equivalent proteins when we immunoblotted using anti-β-actin antibody (lower panel).

3.2. *Mapping of the interaction region between human TB10 and human E-Tmod*

To identify the E-Tmod binding region of TB10, cDNA constructs containing two TB10 deletion mutants were designed as shown in Fig. 2A. These truncated regions were predicted to be predominantly α-helices. One deletion mutant

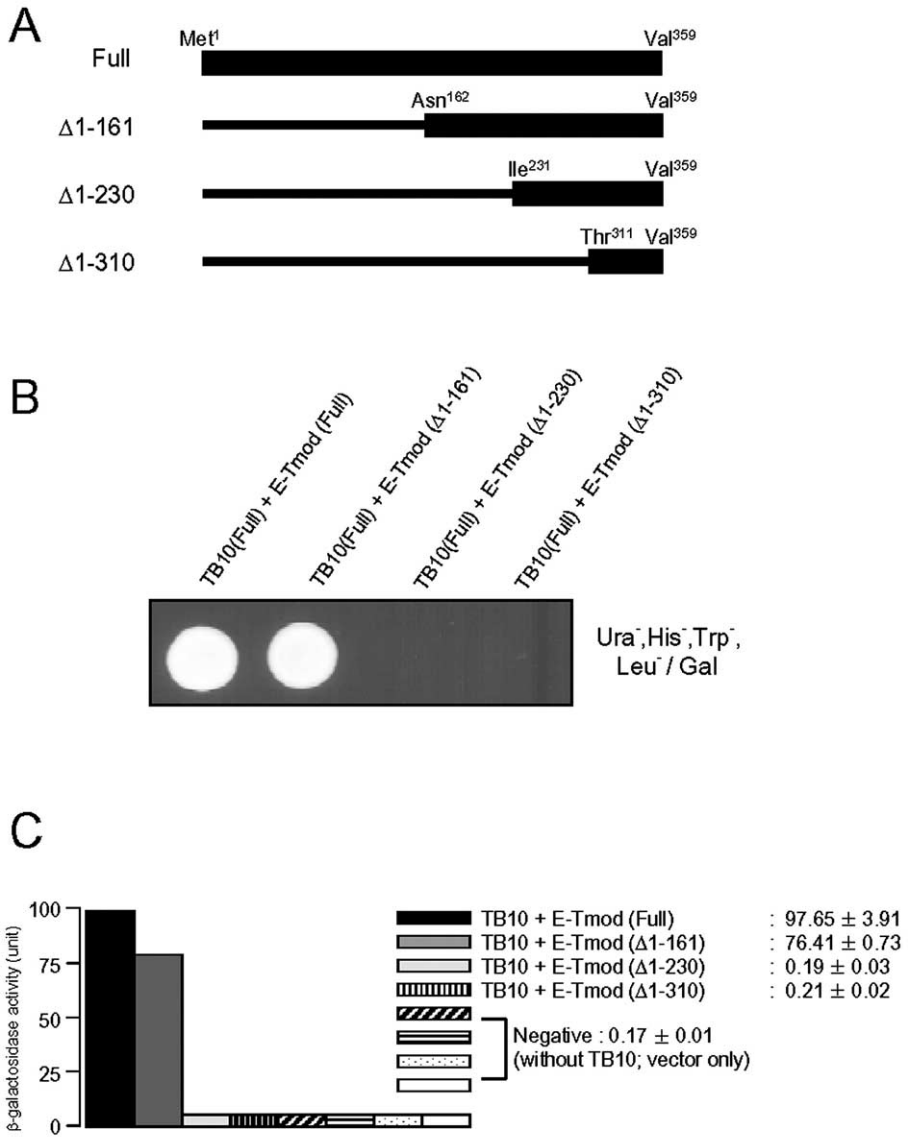

Fig. 3. Mapping of the E-Tmod interaction region with TB10 using the yeast two-hybrid system. A: Schematic representation of cDNA constructs for each human E-Tmod deletion mutant and full-length human E-Tmod fusion proteins in the yeast two-hybrid system. B: cDNA constructs were co-transformed into EGY48 yeast cells to test the protein–protein interaction within the yeast two-hybrid system. C: Binding activity of these constructs by ONPG assays. The results are representative of three separate experiments. Data are shown as mean ± S.E.M.

(Δ-1–28) does not contain the LKKTET motif that is common to β-thymosins. These regions were shown to be responsible for binding actin [12]. Another deletion (Δ29–43) mutant contains the LKKTET motif.

In the two-hybrid system, the full-length human E-Tmod cDNA and either plasmid containing a full-length human TB10 cDNA (Fig. 2A, Full) or plasmids containing two truncation mutant forms (Fig. 2A, Δ-1–28 and Δ29–43) of cDNAs were co-transformed into EGY48 yeast cells. Cells containing only full-length TB10 cDNA construct grew on the Ura, His, Trp and Leu deficient plates, whereas yeast cells transformed with both deletion mutant colonies (Δ-1–28 and Δ29–43) failed to grow (Fig. 2B). To confirm this result, we determined the binding activity of these constructs by measuring the relative expression level of β-galactosidase. As shown in Fig. 2C, β-galactosidase assay results confirmed that either of these mutants cannot bind to E-Tmod. However, we cannot exclude that the E-Tmod binding site of TB10 is located near the junction of two α-helices.

Subsequently, cDNA constructs containing three E-Tmod truncation mutants were designed to localize the TB10 binding region of E-Tmod (Fig. 3A). These truncated regions were predicted to be predominantly α-helices. In the two-hybrid system, full-length human TB10 cDNA and either full-length human E-Tmod cDNA (Fig. 3A, Full) or three truncation mutants (Fig. 3A, Δ1–161, Δ1–230 and Δ1–310) were co-transformed into EGY48 yeast cells. Cells containing full-length E-Tmod cDNA and also the smallest deletion mutant (Δ1–161) grew on the Ura, His, Trp and Leu deficient plates. Yeast cells transformed with the other deletion mutants (Δ1–230 and Δ1–310) failed to grow (Fig. 3B). We also quantitated the binding activity of these constructs by measuring relative expression level of β-galactosidase. Consistent with Fig. 3B, the critical E-Tmod regions for binding TB10 resided within Asn^{162} and

Ser^{230} (Fig. 3C). The β-galactosidase activity for interactions between TB10 and one partial deleted mutant of E-Tmod (Δ1–161) was lower than that of full-length E-Tmod. This may indicate that the region between Met^{1}-Leu^{161} of E-Tmod enhances TB10 binding, but is not critical.

3.3. *E-Tmod rescues tumor cells from TB10 mediated apoptosis by restoring actin architecture*

Since the TB10 binding site of E-Tmod (Asn^{162}-Ser^{230}) partially overlaps with the actin binding site of E-Tmod (Ile^{190} and Val^{359}), we tried to block TB10 mediated apoptosis by introducing the full-length E-Tmod cDNA. SKOV-3 ovarian cancer cell line was grown in DMEM and co-transfected with different ratios of TB10 and E-Tmod cDNAs (Fig. 4A). Three days after transfection, the cells were harvested, stained with trypan blue and quantitated to analyze cell viability. Mock transfectants include the expression vector only and control indicates cells without transfection. Viability of the transfectant containing only TB10 was very low compared to that of the mock transfection or control, indicating that this transfectant undergoes vivid apoptosis (Fig. 4A, left). However, viability of the transfectant containing only E-Tmod was very similar to that of the mock transfectant or control, supporting that E-Tmod did not affect apoptotic process (Fig. 4A, left). Co-transfection with TB10 (5 μg) and E-Tmod cDNA (5 μg) at a ratio of 1:1 significantly decreased apoptosis compared to that of transfectant containing only TB10 (Fig. 4A, right). Furthermore, co-transfection with TB10 (5 μg) and E-Tmod cDNAs (2.5 and 1 μg) at higher ratios of 2:1 and 5:1 correlated with increased apoptosis (Fig. 4A, right). Also, co-transfection with TB10 (2.5 and 1 μg) and E-Tmod cDNAs (5 μg) at ratios of 1:2 and 1:5 gradually decreased apoptosis (Fig. 4A, right). These combined results suggest that E-Tmod expression efficiently blocks TB10 mediated apoptosis.

We also stained with DAPI to confirm that the loss of viability is due to apoptosis. Transfectants containing only TB10 cDNA showed clear DNA fragmentations, whereas either mock transfectants or transfectant containing only E-Tmod cDNA did not show any signs of DNA fragmentation (Fig. 4B). In agreement with cell viability assay, co-transfectant with TB10 and E-Tmod cDNAs at a ratio of 1:1 clearly decreased DNA fragmentation compared to that of transfectant containing only TB10 (Fig. 4B).

Further, we performed phalloidin staining assays on SKOV-3 ovarian cancer cells to analyze the effect of E-Tmod on TB10 mediated apoptosis. As shown in Fig. 4C, staining with phalloidin-FITC revealed the presence of an intact and pervasive actin structure in control and E-Tmod transfected cells, whereas overexpression of TB10 dramatically disrupted the F-actin stress fibers. Strikingly, E-Tmod and TB10 overexpressed cells restore the actin architecture almost similarly to mock and E-Tmod transfected cells. This is clear evidence that the interaction between E-Tmod and TB10 rescues cells from TB10 mediated apoptosis by restoring actin architecture.

4. Discussion

We identified E-Tmod, a component of the actin complex, as a novel TB10 binding protein. The interaction between TB10 and E-Tmod could be the first example of a regulation

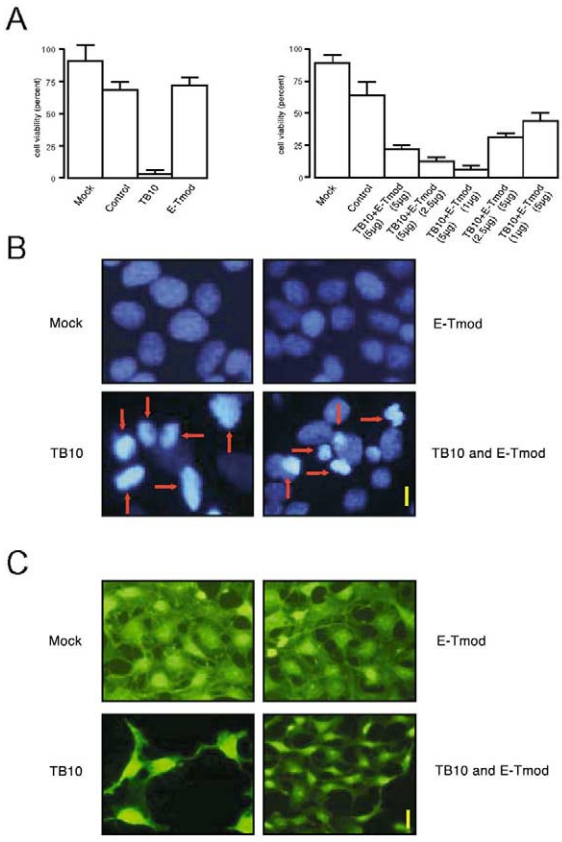

Fig. 4. E-Tmod rescues TB10 mediated apoptosis by restoring actin architecture. Cell viability assay (A), apoptosis assay (B) and phalloidin staining (C) were performed on SKOV-3 ovarian cancer cells transfected with TB10 and/or E-Tmod. The results are representative of three separate experiments. A: Cells were stained with trypan blue and quantitated to analyze cell viability. Data are shown as mean ± S.E.M. B: Cells were stained with DAPI to visualize DNA fragmentations for the apoptosis assay. Arrows indicate the observed DNA fragmentations. Size bar, 20 μm. C: Cells were stained with phalloidin-FITC to visualize F-actin networks. Mock means cells transfected empty expression vector and control means those without transfection. Size bar, 20 μm.

mechanism of thymosin-β proteins which may have a role in preventing apoptosis.

E-Tmod is the P-end (pointed or slow growing end) capping protein that modulates the actin binding proteins to control the length of the actin filament [13–15]. Blocking the E-Tmod capping activity in cultured myocytes results in an abnormal assembly of the actin architecture [13,16].

Biochemical analysis of E-Tmod revealed two functional domains of E-Tmod. One is the N-terminal half that is predicted to be elongated and flexible [17]. The flexible and extended conformation of the N-terminus between Glu^{6} and Val^{184} may be required for the direct interaction of E-Tmod with tropomyosin [18]. The C-terminal half between Ile^{190} and Val^{359} is proposed to be compact and tightly folded and involved in the capping activity to actin [17,19]. Although the details of the E-Tmod–tropomyosin interaction have not been clarified, the interaction site of E-Tmod is dependent on the type of target tropomyosin. Interestingly, the region from amino acids Glu^{6} to Lys^{94} of E-Tmod interacts with skeletal muscle tropomyosin, whereas the region from Glu^{90} to Glu^{184} interacts with erythrocyte tropomyosin [20]. Our results on the E-Tmod binding assay with TB10 using the yeast two-hybrid

system revealed that the critical E-Tmod regions for binding TB10 resided within Asn^{162} and Ser^{230}. This region is partially overlapped with the actin binding site.

To determine which TB10 regions are required for the interaction with E-Tmod, we constructed two deletion mutants and tested them using the yeast two-hybrid system. TB10 is composed of two α-helices and we have tried to test whether two helices are important for its binding with E-Tmod. One deletion mutant (Δ-1–28) does not contain the LKKTET motif that is common to β-thymosins [21]. These regions were shown to be responsible for binding actin [21]. Another deletion (Δ29–43) mutant contains the LKKTET motif. Thus, our results revealed that both helices appear to be required for the interaction.

It has been suggested that the cytoskeleton is involved in the morphological changes associated with apoptosis. Consistent with this, reorganization of the microfilament network is necessary to form apoptotic bodies. In addition, depolymerization of F-actin may also be a necessary component of the apoptotic process. Thus, it is not surprising that actin associated proteins could be involved in the apoptotic process. For example, cleavage of actin modulating proteins such as fodrin and gelsolin by interleukin-I converting (ICE) or ICE-like protease during the apoptotic process presumably alters the homeostasis of the actin polymerization status [22–24].

Herein, we showed another example of an actin binding protein modulating the apoptotic process. The fact that TB10 shares the same binding site for E-Tmod (Asn^{162} to Ser^{230}) as for actin may explain how TB10 overexpressing cells undergo apoptosis. Overexpression of TB10 could bind to E-Tmod and prevent an interaction between E-Tmod and actin. In the absence of available E-Tmod, the actin complex would become unstable, resulting in the disruption of the cytoskeletal architecture. Supporting this idea, we have shown that co-transfection with E-Tmod and TB10 into ovarian cancer cells restores actin architecture, whereas transfection with TB10 alone disrupts the actin architecture. Also, varying the relative ratios of TB10 and E-Tmod cDNA in transfected cells modulates cell viability.

In summary, we have identified E-Tmod as a novel binding partner for TB10 via yeast two-hybrid screening. We have also mapped the critical molecular domains required for this interaction. Finally, transfection experiments indicate that TB10 may bind E-Tmod to prevent the actin filament complex and induce an apoptotic process. These combined results suggest that TB10 may have an important biological role in regulating E-Tmod function and possibly in targeting E-Tmod to the actin complex where it could participate in the regulation of the apoptotic process.

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